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Levels of Antibody to Defined Antigens of *Mycobacterium tuberculosis* in Tuberculous Meningitis

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The aim of the study was to develop an antibody immunoassay for tuberculous meningitis with a sensitivity greater than that of the initial bacterial smear while maintaining a 100% specificity. Antibody titers to five purified antigens of *Mycobacterium tuberculosis* were measured in cerebrospinal fluid by using an enzyme-linked immunosorbent assay technique. Seventy-four patients with tuberculous meningitis (26 culture positive) were compared with 26 patients with purulent meningitis, 69 patients with suspected but excluded tuberculous meningitis, and 29 patients with other neurological diseases. Antibody responses to both the 14-kilodalton (kDa) antigen and lipoarabinomannan were immunodominant, accounting for 74% of those with any antibody titer to a soluble extract of *M. tuberculosis*. A sensitivity of 61% was achieved (77% for the culture-positive samples) by using immunoglobulin G titers to lipoarabinomannan and the 14-, 19-, and 38-kDa antigens. Thus, estimation of levels of antibody in cerebrospinal fluid to selected mycobacterial antigens would be valuable in the diagnosis of tuberculous meningitis. The possible reasons for the immunodominance of the 14-kDa antigen and lipoarabinomannan in the pathogenesis of tuberculous meningitis are discussed.

Tuberculous meningitis (TBM) is an important form of extrapulmonary tuberculosis in the Indian subcontinent and is associated with a significant mortality (19, 20). Despite the decline in the incidence of TBM in the United Kingdom, it remains a diagnostic problem, particularly in children, the elderly, and immigrants from the Indian subcontinent (12, 16, 17). Presenting symptoms are nonspecific, and the diagnosis may remain unsuspected until signs of meningeal irritation appear. Examination of the cerebrospinal fluid (CSF) may reveal lymphocytosis together with high protein and low glucose concentrations, although these characteristics may be found to be associated with other diseases such as viral meningitis and partially treated purulent meningitis (PM); and occasionally, the CSF may even appear normal (15, 21). Delay in the diagnosis of TBM is directly related to a poor prognosis, while early treatment permits recovery without neurological sequelae (12). A simple diagnostic test which can reliably identify patients with TBM from those with other diseases whose clinical appearance resembles TBM would be an invaluable adjunct to standard microbiological techniques. Such a test would need to have a high specificity and a sensitivity greater than that of the initial smear (5).

Antibody titers in CSF to crude extracts of *Mycobacterium tuberculosis* have been observed in patients with TBM (2). However, the cross-reactivity of mycobacterial antigens has given rise to false-positive results in immunoassays, whether antigen or antibody was detected (for a review, see reference 5). A lipopolysaccharide, lipoarabinomannan (LAM), which is peculiar to mycobacteria, has been chemically purified in quantities that permit its use as a diagnostic reagent (9, 13). Monoclonal antibodies (MAbs) directed against antigenic determinants that are highly specific for tubercle bacilli have been used to identify protein antigens which have subsequently been prepared from recombinant clones or purified by affinity chromatography from culture filtrate (11). It has been suggested that the species specificity (restriction) of antigenicity of mycobacterial species almost certainly rests not with whole antigens but with their epitopes (6). However, the serological response to the individual epitopes defined by such MAbs correlated well with the total immunoglobulin G (IgG) binding for LAM and the 14- and 38-kilodalton (kDa) proteins, suggesting that the species-specific epitopes on these antigens are immunodominant (8, 11). In this study, LAM and four purified antigens recognized by MAbs that distinguish tubercle bacilli from nontuberculous mycobacteria were used in a simple direct binding enzyme-linked immunosorbent assay to measure antibody titers in CSF.

MATERIALS AND METHODS

Patients. Samples of CSF from 195 patients were collected after routine cell count, cytology, India ink preparation for fungal protein, glucose measurement, and microbial culture, including that for mycobacteria. Twenty-six patients were shown to have TBM after culture of *M. tuberculosis* from their CSF (TBM group); in five of these patients, tuberculomata were demonstrated by a computed tomography scan or a subsequent postmortem examination. A clinical diagnosis of TBM was made on the basis of CSF findings in the absence of culture of tubercle bacilli and with evidence supplied by the clinical history, radiography, a failure to grow organisms after bacterial culture, and symptomatic improvement with antituberculous chemotherapy in 48 patients (clinical TBM group); this group was intentionally greater in proportion compared with the culture-positive TBM group in order to highlight any increase in sensitivity obtained with the antibody test; the relative proportion does not reflect the detection rate of TBM by microbiological culture. CSF was also obtained from 26 patients with PM, 69 patients in whom a diagnosis of TBM was compatible with the presenting clinical features but in whom an alternative diagnosis was achieved (suspected but excluded TBM [SEM group]; this included 16 patients with viral meningitis), and

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TABLE 1. Diagnosis of patients

Diagnosis	No. of cases
TBM	74
Bacteriologically confirmed	26
Clinically diagnosed	48
PM	26
Suspected but excluded TBM (SEM)[a]	69
Viral meningitis or encephalitis	16
Cerebrovascular disease	16
Neoplasia: carcinomatous meningitis, metastatic carcinoma, glioma, other	11
Cysticercosis	9
Degenerative: dementia, cerebellar degeneration	7
Others: lupus encephalitis, leukodystrophies, diabetic ophthalmoplegia, Reye syndrome, neurosyphilis, psychosis	10
OND[b]	29
Structural: congenital hydrocephalus, cervical spondylosis, prolapsed intervertebral disk	12
Other: Parkinson's disease, idiopathic epilepsy, peripheral neuropathy, motor neuron disease, multiple sclerosis	17

[a] Patients in whom a diagnosis of TBM was compatible with the presenting symptoms.
[b] Patients in whom the presenting symptoms suggested a diagnosis other than TBM.

29 patients whose presenting symptoms suggested a diagnosis other than TBM (other neurological diseases [OND]; Table 1).

Antigens. A standard soluble extract (SE) of *M. tuberculosis* H37Rv bacilli was prepared as previously described (10). LAM was chemically purified as described previously (9). The 14-, 19-, and 38-kDa antigens were obtained by affinity chromatography of the *M. tuberculosis* H37Rv culture filtrate by using MAbs TB68, TB23, and TB71; mouse antibody fragments were removed by passing them through a column coupled with rabbit anti-mouse immunoglobulin (11). The recombinant 65-kDa antigen was a kind gift from J. van Embden (National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands [18]).

Enzyme-linked immunosorbent assay. Microtiter plates (Immulon; Dynatech, Billingshurst, Sussex, United Kingdom) were coated with *M. tuberculosis* SE at 10 μg/ml, the 14- and 19-kDa antigens were coated at 0.2 μg/ml, the 38-kDA protein was coated at 0.35 μg/ml, the 65-kDA protein was coated at 1 μg/ml, and LAM was coated at 0.1 μg/ml by overnight incubation at 4°C. These coating concentrations were determined by plateau binding to their respective MAbs. A sample blank was performed by coating it with phosphate-buffered saline. After the plates were blocked with 1% dried milk (Sainsbury plc, London, United Kingdom) in phosphate-buffered saline containing 0.05% (vol/vol) Tween 20 (Sigma Chemical Co., Poole, Dorset, United Kingdom) for 30 min at 37°C, serial dilutions of CSF (1:5, 1:25, 1:125, and 1:625) were incubated for 1 h at 37°C. The plates were washed and incubated with peroxidase-labeled, affinity-purified goat anti-human γ or μ heavy chains (Sigma) for 1 h at 37°C, washed, and then developed with tetramethyl benzidine tetrahydrochloride (0.1 mg/ml; Aldrich, Gillingham, Dorset, United Kingdom) in 0.1 M citrate buffer (pH 5) containing 0.01% H_2O_2 for 10 min; the reaction was stopped by the addition of acid, and the A_{450} was measured. Antibody titers were expressed as that dilution of CSF giving 30% of the count of a standard hyperimmune serum sample.

Statistics. Statistical analysis was performed as described previously (1). Antibody titers were compared by using a Wilcoxon two-sample test (Mann-Whitney test) or a k sample generalization of this test (Kruskal-Wallis analysis) by using the Minitab statistical package (T. A. Ryan, Jr., Pennsylvania State University, University Park). Age groups were compared by using Student's t test. Correlation coefficients were calculated with 95% confidence limits by using the Spearman rank test. Specificity and sensitivity were calculated from the results that were obtained.

RESULTS

Antibody levels were higher in the 26 patients in whom TBM was confirmed by culture of *M. tuberculosis* than they were in the other 48 patients in whom a diagnosis of TBM was made on clinical criteria alone (Table 2). In the PM, SEM, and OND control groups, antibody levels to all the purified antigens except the 65-kDa antigen were barely detectable. The mean antibody titer to the 65-kDa protein in patients with PM was similar to that found in patients with clinically diagnosed TBM and was matched by anti-*M. tuberculosis* SE antibody levels of the same magnitude.

Comparison of antibody titers in patients with TBM, PM, SEM, and OND by the Kruskal-Wallis test showed a significant variation for all antigens tested ($H > 9.5$; $P < 0.05$ for 4 degrees of freedom; Table 2). IgG titers to purified LAM

TABLE 2. Comparison of antibody levels in the different patient groups

Patient diagnosis	Immunoglobulin class	Specificity ($\log_5$ mean antibody titer ± SD) of the following antigens[a]:					
		M. tuberculosis SE	LAM	14 kDa	19 kDa	38 kDa	65 kDa
TBM (n = 26)	IgM	1.20 ± 1.10**	0.55 ± 0.92*				
	IgG	2.40 ± 1.47**	1.16 ± 1.23**	1.08 ± 1.33**	0.27 ± 0.57	0.16 ± 0.57	0.87 ± 1.09**
Clinical TBM	IgM	1.09 ± 0.99**	0.51 ± 0.88*				
(n = 48)	IgG	1.81 ± 1.38**	0.90 ± 1.25**	0.48 ± 0.86*	0.28 ± 0.57	0.13 ± 0.44	0.48 ± 0.86*
PM (n = 26)	IgM	0.24 ± 0.79	0.09 ± 0.47				
	IgG	0.49 ± 0.86	0.00 ± 0.00*	0.03 ± 0.17	0.02 ± 0.08	0.00 ± 0.00	0.44 ± 0.97
SEM (n = 69)	IgM	0.05 ± 0.20**	0.04 ± 0.24*				
	IgG	0.13 ± 0.42**	0.03 ± 0.15**	0.00 ± 0.00**	0.02 ± 0.11	0.00 ± 0.00	0.07 ± 0.33**
OND (n = 28)	IgM	0.00 ± 0.00**	0.00 ± 0.00				
	IgG	0.00 ± 0.00**	0.00 ± 0.00*	0.00 ± 0.00*	0.01 ± 0.08	0.00 ± 0.00	0.00 ± 0.00*

[a] *, $P < 0.05$; **, $P < 0.01$; significance of the z value, which measures the departure of the group mean ranked titer from the overall ranked titer. For the Kruskal-Wallis analysis, the H value estimates the degree of variation in the tested groups (equal to the χ^2 value for $k - 1$ degrees of freedom; $H > 9.5$ is significant at $P < 0.05$). The H values for IgM to *M. tuberculosis* SE and LAM were 99.6 and 121.1, respectively, and for IgG were 32.5 and 67.3, respectively. The H values for IgG to the 14-, 19-, 38-, and 68-kDa antigens were 68.8, 13.3, 16.6, and 37.8, respectively.

TABLE 3. Antibody (IgG) positivity in the CSF of patients and controls

Patient group	Test antigen	% of subjects with IgG titer of:					% Positive[a]
		0	1–4	5–24	25–124	≥125	
Controls (n = 98)[b]	MTSE[c]	93	2	7	0	0	7
	LAM	97	3	0	0	0	0
	14 kDa	100	0	0	0	0	0
PM (n = 26)	MTSE	69	4	27	0	0	27
	LAM	100	0	0	0	0	0
	14 kDa	96	4	0	0	0	0
TBM (n = 74)	MTSE	14	15	15	35	22	72
	LAM	50	8	22	12	8	42
	14 kDa	58	16	15	7	4	26
	LAM, 14 kDa						53[d]
	LAM and 14, 19, and 38 kDa						61[d]

[a] Titers of >5.
[b] Combined SEM and OND clinical groups.
[c] MTSE, *M. tuberculosis* SE.
[d] Positive rating significantly greater than culture results (χ^2 test; $P < 0.05$).

and the 14-kDa antigen showed the greatest discrimination between TBM patients and controls (H values, 67.3 and 68.8, respectively, with highly significant departures from the overall mean rank, as measured by the z values). However, despite a significant variation in the antibody titers for the 19- and 38-kDa antigens among the five groups (H values, 13.3 and 16.6, respectively), the distribution did not permit good discrimination between those with TBM and those with PM, SEM, and OND (no z value demonstrated a group whose mean rank differed significantly from the overall mean rank). While levels of IgG antibody to *M. tuberculosis* SE could statistically distinguish between patients with TBM and controls, high titers in a few patients with SEM, OND, and PM reduced the diagnostic benefit when compared with measurement of antibodies to LAM and the 14-kDa protein (Table 3).

Levels of IgG antibody to *M. tuberculosis* SE in patients with TBM showed significant correlations with titers to LAM and the 14-, 19-, and 65-kDa antigens ($r = 0.51 \pm 0.09$, 0.49 ± 0.09, 0.35 ± 0.10, and 0.42 ± 0.10, respectively; $P < 0.01$ for all groups). However, in patients with PM the levels of IgG binding to the 65-kDa antigen were the only ones to show any correlation with binding to *M. tuberculosis* SE ($r = 0.41 \pm 0.16$; $P = 0.03$). While antibody binding to the 65-kDa antigen may have accounted for some of the cross-reactive humoral response in the CSF of patients with PM, three patients exhibited high *M. tuberculosis* SE antibody titers without any corresponding binding to the 65-kDa protein.

LAM is a polysaccharide in which antigenic determinants consist of repeating 5-linked D-arabinofuranose residues (9) and might therefore be expected to stimulate a pronounced IgM response, unlike the protein antigens that were tested. However, while IgM titers to *M. tuberculosis* SE could distinguish patients with TBM from those in the SEM and OND groups, the variation in LAM titers was less discriminatory (Table 2). One patient in the PM group and one patient in the SEM group showed IgM binding to LAM with a titer of >5. Of the 33 patients with TBM with an IgM antibody titer to *M. tuberculosis* SE of >5, only 16 (48%) showed a corresponding IgM response to LAM; IgM titers to *M. tuberculosis* SE and LAM were found as frequently in culture-positive patients with TBM as in patients with clinical TBM. Even so, three patients with TBM showed an IgM humoral response in CSF to *M. tuberculosis* SE with no accompanying IgG antibody titer, and two of these three displayed IgM titers to LAM.

Only nine CSF samples, all from patients with TBM, had any measurable binding activity to the 38-kDa antigen. It is of interest that eight of these nine patients had evidence of pulmonary tuberculosis, in which elevated anti-38-kDa titers represent the most immunodominant specificity (11).

The sensitivity of the combined positive titers to LAM and the 14-kDa antigens was 53%, and the sensitivity to all purified antigens except for the 65-kDa antigen was 61%. Both of these values are significantly greater than those obtained by standard microbiological techniques, including the results of culture of *M. tuberculosis* ($\chi^2 = 4.0$ and 8.8; $P = 0.04$ and 0.003, respectively), while they maintained a specificity of 100% (Table 3). In patients with TBM who had measurable antibody titers to *M. tuberculosis* SE, levels of antibody to LAM and the 14-kDa antigen in CSF were positive more frequently (39 of 53 patients; 74%) than those to the 19- and 38-kDa antigens (14 of 52 patients; 27%) and contributed most (53 of 61 patients, i.e., 87%) to the sensitivity of the assay in detecting TBM without false-positive results in the PM, SEM, and OND control groups (Table 3). While levels of antibody (IgG) to LAM and the 14-kDa antigens showed a significant correlation ($r = 0.42 \pm 0.10$; $P = 0.001$), about one-third (7 of 20 samples) of CSF samples showed IgG binding to the 14-kDa antigen without any detectable binding to LAM, and 28% (9 of 32 patients) of patients with TBM showed antibodies to LAM without any detectable binding to the 14-kDa antigen. Those with antibody titers to the 14-kDa antigen alone were significantly younger than those with titers to LAM alone (Student's t test; mean age, 16.9 years [range, 1 to 40 years] versus 30.9 years [range, 15 to 50 years]; $t = 2.2$; $P = 0.04$). Although the diagnostic sensitivity of LAM was the better of the two antigens, the combined results with the 14-kDa antigen significantly improved the sensitivity of the antibody assay in the CSF from those in the younger age group. Samples of CSF from all five patients with tuberculomata contained antibody levels to the 14-kDa and LAM antigens similar to those in the other patients with TBM. Only one patient with PM demonstrated a low antibody titer to the 14-kDa antigen; excluding this patient, the discrimination of patients with TBM would have been identical to that achieved with LAM (42%), with no false-positive results obtained for the SEM and OND groups.

Only a small fraction of patients with TBM had antibody titers to *M. tuberculosis* SE of >5 without demonstrating a positive titer to one of the five antigens tested (8 of 74 patients; 15% of positive titers to *M. tuberculosis* SE). However, a similar proportion of the patients with PM (3 of 26 patients) and a smaller number of control CSF samples (5 of 98 samples) showed titers to *M. tuberculosis* SE without corresponding positive titers to any of the tested antigens.

DISCUSSION

The antibody test for CSF described here was based on an enzyme-linked immunosorbent assay in which accessible antigens were used. LAM has been purified in quantity by chemical means (9), while the 14-kDa protein has been expressed by λgt11 recombinant DNA clones (23, 25) and could be overproduced in alternative vectors and then purified, as has already been achieved with the 65-kDa protein (18). Since other reagents are readily obtainable and the test can be completed within 4 h with precoated plates, the technique can be used easily in countries in which TBM is common.

The relative immunodominance of antibody formation to LAM, which was demonstrated previously by Western blotting (immunoblotting) (4), was confirmed in this study. Detection of LAM antigen by reverse passive hemagglutination with erythrocytes coated with an IgM MAb (ML34) produced false-positive results in control CSF samples from humans living on the Indian subcontinent (4). Samples of CSF in the same diagnostic categories as those which gave false-positive results before showed, in the present study, no binding of IgG antibody to LAM and only a single false-positive IgM titer in the OND group. Indeed, no detectable anti-LAM IgG antibody was found in samples of CSF from patients without TBM in which known false-positive results were obtained by the methods of the previous study (4) (data not shown). On the other hand, measurement of levels of IgM antibody to LAM in CSF was disappointing, with two false-positive results, and only two samples from patients with TBM in which a positive result was not associated with IgG titers.

The 14- and 38-kDa protein antigens both have epitopes defined by MAbs that are confined to tubercle bacilli, unlike all other proteins that have been identified so far with MAbs specific for mycobacteria (7). In this study levels of IgG antibody to the 14-kDa protein contributed to the diagnosis of TBM, particularly in patients who did not have anti-LAM IgG in the CSF; such patients were younger compared with those with IgG antibodies to LAM but not to the 14-kDa protein. Whether the differential response to LAM and the 14-kDa antigen could be a marker for different pathological processes in patients with tuberculosis needs to be explored. In children, TBM may arise as a result of progressive primary disease, while in older people, smear-positive pulmonary disease and the subsequent hematogenous spread to the meninges appears to be more common (14). However, compared with other patients with TBM, the CSF of patients with tuberculomata did not contain a distinct antibody pattern.

It is of interest that antibody titers to the TB68 epitope of the 14-kDa antigen in serum were shown to be higher following *Mycobacterium bovis* BCG vaccination in 10% of children with tuberculous mediastinal lymphadenitis, in patients with self-healed tuberculosis, and in healthy hospital staff who were in contact with patients with tuberculosis (3, 8). Antibody titers to the TB68 epitope, but not to the whole 14-kDa antigen, in serum from patients with TBM were also higher than those in serum from controls (data not shown). Antibody levels to the 38-kDa protein have been detected previously only in the serum of patients with smear-positive but not smear-negative pulmonary tuberculosis (11), and the low sensitivity of the 38-kDa in patients with TBM further supports a distinct role of this antigen in the development of smear-positive pulmonary tuberculosis.

The 65-kDa antigen, which belongs to a class of stress proteins, has been shown to have epitopes that cross-react with most bacteria (22, 24). Consequently, the observed false-positive rating in patients with PM, and indeed, with diseases other than TBM, was expected. While antibody to the cross-reactive 65-kDa antigen could have partly accounted for the antibody levels to *M. tuberculosis* SE, 5% of controls had anti-*M. tuberculosis* SE antibody titers that were not attributable to binding to any of the purified antigens that we tested. Although the CSF from 11% of patients with TBM contained antibodies to additional, but as yet unidentified, antigens of *M. tuberculosis*, these antibodies appeared unlikely to raise considerably the diagnostic sensitivity. However, more than half of the CSF samples from patients with TBM demonstrated IgM binding to *M. tuberculosis* SE without detectable IgM antibody to LAM, suggesting that antigens other than LAM might induce IgM responses of potential diagnostic significance. Nevertheless, we conclude that IgG antibodies in CSF to LAM and the 14-kDa protein, which confirmed the diagnosis of TBM with a 53% sensitivity and 100% specificity, offer a valuable predictive test which deserves to be added to standard techniques in microbiological laboratories.

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